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The aim of the research program we are developing is to define molecular markers and their interaction with other risk factors as risk indicators for development of breast cancer among women with benign breast disease (BBD). Our specific aims are:

1. Estimate the incidence and time span of breast cancer development in a large cohort of African American and Caucasian women with biopsy-proven BBD;

2. Collect and archive in a specimen bank samples of benign breast disease lesions and breast cancer from women in this cohort;

3. Develop and test a questionnaire for collecting breast cancer risk factor information that will:
   a) allow the construction of an exposure index for lifetime exposure to sex hormones; and
   b) designed to be sensitive to the perceptions of African American as well as Caucasian women.

We are constructing a cohort of 5353 women with BBD between 1981-1991, who will be followed from 5-15 years and yield 248 women who will have developed invasive breast cancer. This work is building the foundation, in terms of a cohort, a specimen bank, a survey instrument, and a summary information index, for the conduct of molecular epidemiologic studies of breast cancer.

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Christine Cole Johnson 10-10-97

PI - Signature Date

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INTRODUCTION

The Specific Aims have not been modified from the original proposal. The eventual aim of the research program we are developing is to define molecular markers and their interaction with other epidemiologic risk factors, particularly exposure to sex hormones, that can serve as risk indicators for subsequent development of breast cancer among two groups of women with benign breast disease (BBD), Caucasians and African Americans. Our work this past year and in the year to come is laying part of the foundation for our long term research goals. Our specific aims for this developmental work are:

1. to estimate the incidence and time span of breast cancer development in a large cohort of African American and Caucasian women with biopsy-proven BBD;

2. to collect and archive in a specimen bank samples of benign breast disease lesions and breast cancer from women in this cohort;

3. to develop and test a questionnaire for collecting breast cancer risk factor information that will:

    a) allow the construction of an exposure index for lifetime exposure to sex hormones; and

    b) designed to be sensitive to the perceptions of African American as well as Caucasian women.

This work will provide the foundation for a future research program planned to use the established cohort, biorepository and data collection instruments to delineate clinically important molecular biomarkers of risk and progression in breast cancer and to provide further molecular discriminators of risk in addition to other correlates such as histologic parameters, estrogen and progesterone exposure, reproductive history, family history of breast cancer, and various demographic characteristics. The

important clinical and public health implications of this study include: 1) the ability to identify women with high risk lesions and/or personal characteristics who then can be carefully followed; 2) the ability to identify and reassure a larger population of women having lesions with no increased risk; and 3) the ability to correlate DNA markers, DNA ploidy and histology with hormonal and familial risk factors.

Body

Women with benign breast lesions, particularly those with lesions classified as proliferative, are at increased risk for subsequent development of breast cancer. The eventual aim of the research program we are developing at Case Western Reserve University--Henry Ford Health Sciences Center, is to define molecular markers and their interaction with other epidemiologic risk factors, particularly exposure to estrogen, that can serve as risk indicators for subsequent development of breast cancer among two groups of women with benign breast disease (BBD), Caucasians and African Americans. This application is to accomplish preliminary work that would lay part of the foundation for the eventual research program and be generally applicable in the field of breast cancer epidemiology as well.

The information we gain from this proposed work will be used in an eventual study to evaluate, within the identified cohort and using a nested case-control approach, histopathological, molecular, and personal characteristics, including an hormone exposure index, and their interactions as risk factors for the development of breast cancer among African American and Caucasian women with biopsy proven BBD. The developed questionnaire would be useful in general in the conduct of epidemiologic studies of breast cancer, especially those that include African American women. If we are successful in developing an index, the model could be adjusted as new biological information is acquired regarding the relationship of reproductive characteristics and body burden of estrogen and progesterone, and could be used in future work. The hormone exposure index could be used to re-evaluate data from previous breast cancer risk factor studies to examine whether a continuous summary score might better explain case-control status.

Experimental Methods

1.0 Specific Aim 1: Cohort Establishment and Follow-up

1.1 Cohort Enrollment

Subjects are being obtained from patients who underwent breast biopsy at HFHS in Detroit, MI from 1981-1991. The patients at HFHS are largely from the three-county metropolitan Detroit area. Each pathology report in the Department of Pathology patient files dated January 1981 through December 1991 is being reviewed by a trained research assistant. As an estimate, approximately 28,000 reports were filed in 1980 and 66,000 in 1986 (from 1988 -1991 this search will be facilitated through the use of a computerized data base). The research assistant is identifying all cases with a breast biopsy and pulling and copying the pathologic reports. Dr. Raju, (rather than the Co-PI, Dr. Worsham, as originally planned) is reviewing the copies of the pathology reports and identifying the biopsies with a diagnosis of BBD. (Drs. Zarbo and Wolman are consulted for ambiguous cases. Women with a concurrent or previous invasive carcinoma in the same breast or contralateral breast are excluded as they cannot be considered wholly "disease free" (at risk) upon entry into the cohort. (Women with a diagnosis of carcinoma in situ are being set aside as a subset to study in another future project). Individuals who are found to have a diagnosis of breast cancer within six months of the study biopsy are excluded from the cohort as prevalent cases. This is an arbitrary, but customary cut off that presumes such cancers were present at the time of biopsy. When multiple biopsies belonging to one individual are encountered, the first biopsy during the study time period is used, and the date of that biopsy is the time of study enrollment.

The number of eligible subjects with benign tumors is anticipated to be approximately 5350 (Table 1). This number is based on review of available material for 1981 and on data from the computerized data base available from 1988-1991. At HFHS, in accordance with departmental policy, all pathology material dating from 1981 has been saved.

All cases of benign breast disease identified through this procedure will be enrolled in the cohort. All individuals enrolled as study subjects are being followed for occurrence of breast cancer.

Table 1. BBD Study Estimates, Follow up through 12-31-96

Year of BBD	No. BBD Samples	No. Excluded	No. Eligible Subjects	Years of Follow-up	Rate Applied per 100,000 Dx at HFH†	PY Follow-Up	Exp No. HFH Br. C	Total Cases ‡
1981	168	19	149	15	336	2235.24	7.5	10
1982	242	27	215	14	336	3005.16	10.1	13
1983	268	30	238	13	336	3090.31	10.4	14
1984	186	21	165	12	336	1979.78	6.7	9
1985	298	34	264	11	336	2907.59	9.8	13
1986	378	43	335	10	336	3352.86	11.3	15
1987	600*	68	532	9	551	4789.80	26.4	35
1988	821	93	728	8	551	5825.82	32.1	43
1989	740	84	656	7	551	4594.66	25.3	34
1990	840	95	745	6	551	4470.48	24.6	33
1991	887	100	787	5	551	3933.85	21.7	29
	5428	613	4815			40186	186	248

† Actual 1981 rate used for 1981-1986; actual average annual rates from 1988-93 used for 1987-1991.
‡ Based on the 1981 pilot cohort showing a third of cases of breast cancer diagnosed outside HFHS.
* Estimate, other years actual.

1.2 Cohort Follow-up

The initial source for follow-up information is the Henry Ford Health System (HFHS) tumor registry. Many of the subjects who develop breast cancer, who continue to reside in metropolitan Detroit, are likely to return to HFHS for diagnosis and treatment. Information stored in the HFHS tumor registry includes basic demographics, in addition to occupation, family history of cancer, and a summary of concurrent and underlying medical conditions.

A second means of case ascertainment will be the state cancer registry. (We originally were going to utilize the Detroit area SEER registry, but the state registry includes all the SEER data as well as cancer cases residing throughout the state of Michigan.) Our study files will be linked with this registry to identify subjects in the cohort who were diagnosed with breast cancer at other institutions. Information

that can be gained from the state registry on identified breast cancers includes histology, grade and stage of cancer, date of diagnosis, and patient's vital status, as well as the place of diagnosis and treatment.

Originally, the research assistant was going to order the medical records for each woman in the cohort who was not identified with an incident breast cancer, and collect all information useful for follow-up purposes e.g., names, addresses and phones numbers of relatives and friends, social security numbers, occupation and employers for the subject and other contacts, insurance, primary and referring physicians, religious preference, date and location of death if applicable, date of last contact, and discharge destination. However, we have found that most of this information is automated in our electronic medical record system, so we are utilizing that source initially to conduct follow-up. All women entered into the study and the next of kin of those known to be deceased, are being contacted through letter and follow-up phone call requesting information on cancer history and a locator form for future contacts. The purpose of this survey is to make certain that cohort members not identified through the various tumor registries have not developed breast cancer, and to update address information.

Subjects or their next of kin who have had a breast cancer diagnosed at a facility that is not affiliated with HFHS are being asked to sign a release document that gives us permission to obtain and review their hospital records to obtain specific information on the reported cancer and obtain pathological material. The records will be abstracted by the data manager and Dr. Johnson, with Drs. McCarthy and Nathanson as consultants.

For subjects lost-to-follow-up, tracing and case ascertainment efforts will utilize a variety of sources including the U.S. Post Office, Michigan Department of Motor Vehicles, religious and professional organizations, local and suburban telephone directories, and reference books available from the library such as the Detroit Criss-Cross Directory. This latter directory can be used to locate names and phone number of neighbors who can be a valuable source for subject re-location information.

Names of individuals who cannot be located with these methods will be submitted to a firm specializing in the tracing of persons for research purposes. This firm, Equifax, has been utilized previously by these researchers with satisfactory results. For each name submitted, the firm provides last known address and vital status. If the individual is found to be deceased, the state from which a death certificate may be obtained is given. (In the future, all death certificates will be requested and the underlying cause of death will be coded along with any other indications of a history of breast cancer. The presence of breast malignancies tends to be reported accurately on death certificates; in one study, each case of breast cancer reported on a sample of death certificates was confirmed by autopsy. It is beneficial to begin this process soon after initial and subsequent attempts to contact each subject have been made since the firm's results can greatly decrease the amount of person-hours needed to search manually for individuals who are difficult to find. The firm will continue to search for pertinent information on those subjects not found through these attempts by linking names on nationwide credit and motor vehicle record files.

1.3 Sample Size and Analysis Plan

There will be data on approximately 4500 women diagnosed with benign breast disease during the years 1981 through 1991 in this study. Our primary objective is to estimate the incidence of breast cancer in this cohort and to model time to breast cancer detection. We assumed that the number of breast cancer cases follows a Poisson distribution and used that distribution to estimate the precision of the estimates. In the table below are the 95% confidence intervals we may expect to obtain for incidence rates ranging from 0.05 to 0.001 and for person years of follow-up ranging from 40,000 to 1,000.

Expected 95% Exact Poisson Confidence Intervals

Incident Breast Cancer Cases

	Per 1000 Person Years of Follow-up			
Person Years of Follow-up	50	10	5	1
40,000	.048, .052	.009, .011	.004, .006	.0007, .0014
20,000	.047, .053	.009, .011	.004, .006	.0006, .0015
10,00	.046, .055	.008, .012	.004, .007	.0005, .0018
1,000	.037, .066	.005, .018	.002, .012	.00003, .0056

We will use Kaplan-Meier curves to describe time to detection of breast cancer adjusting for importance covariates such as ethnicity and BBD diagnosis.

2.0 Specific Aim 2: Identification and Archival of Breast Tissue Specimens

We are establishing a Breast Tissue Biorepository for the pathological material collected from archived samples in this study. Issues to be considered include the design of the physical facility, provision for back-up storage, provision for a quality assurance program, the creation and management of the archival system database including documentation of the storage history for every specimen, specimen deposit and withdrawal policies, specimen "ownership" policies, as well as the practical concerns such as optimal storage containers, type of freezer, and specimen stability.

Dr. Worsham (Co-PI), as Director of the Molecular Epidemiology of Cancer Laboratory (MEC), is overseeing the Breast Tissue Biorepository. The pathology archives will be searched by the laboratory research assistant to retrieve slides and respective paraffin-embedded tissue blocks. When only blocks remain, the blocks will be cut and new slides prepared for storage.

Progress for Aims 1 and 2

1.1 Cohort Establishment

Cohort subjects are being enrolled from the Henry Ford Health System (HFHS) 1981-1994 breast biopsy patient population. To date, breast biopsy pathology reports dating from January 1981 to January 1989 have been retrieved from the pathology patient files. All reports are categorized into benign and malignant specimens by a pathologist with expertise in breast histology, for which we developed a tracking form (included in the Appendix). Two hundred ninety-one breast biopsy specimens from 1981 and 340 from 1982 have been identified as potentially benign. We are currently conducting the categorization of all 1984 breast biopsy reports.

Data bases have been developed that include study ID, medical record number, pathology specimen number, and tracking form results, as well as other data sources (pathology classification, medical record abstract, follow-up information, risk factor questionnaire, tumor registry).

For each potential benign breast specimen, the primary pathologist microscopically reviews all corresponding pathology slides and diagnostically records all lesions on a detailed Pathology Review Form (PRF) which we developed this past year (see Appendix). Our primary pathologist has reviewed a total of 381 specimens (296 from 1981 and 85 from 1982). An intra-rater reliability study has been incorporated into the pathology review, whereby a 10% sample from each cohort year is selected by the programmer for blinded rereview by the primary pathologist. Cases diagnosed with atypical hyperplasia are also reviewed by secondary pathologists for inter-rater reliability (nine cases have been completed to date). One of our biggest hold ups in this work is the time needed for pathology review is longer than anticipated. To address this issue we have delegated as much of the paperwork as possible away from the pathologist, and are considering the possibility of having a pathology resident screen and remove the straightforward cases.

1.2 Cohort Follow-up for Breast Cancer Development

All BBD cohort subjects are being followed for the development of subsequent breast cancer. We are using HFHS and state cancer registries to identify breast cancer cases as well as standard follow-up techniques. Individuals who are found to have a diagnosis of breast cancer within six months of the study biopsy are excluded from the cohort as prevalent cases. We have acquired an automated listing of 1981-1996 diagnosed breast cancer cases (n=3908) from the HFHS Tumor Registry. We are using these data to identify cohort subjects who were later diagnosed with breast cancer in HFHS or who sought cancer treatment within our medical facility. The pathology report date of cancer subjects is being compared against their cancer diagnosis date to determine the time frame between BBD and cancer diagnosis. To date we have run the 1981 BBD cases against the cancer registry data and found 13 cases, 5 of whom were excluded as their breast cancer diagnosis was within 6 months of the BBD biopsy.

We have prepared and submitted a request to the Michigan Department of Community Health to run our study subjects against the state cancer registry for matches. We have developed a letter and locator form (see Appendix) and a trained interviewer has recently begun following up and contacting cohort members to ascertain the occurrence of breast cancer and the willingness of cohort members to participate in a telephone interview at some later point in time. We are also requesting cases or the next of kin of deceased cases diagnosed at other institutions to complete a written informed consent allowing us to obtain and review the slides and take a sample from archived tissue blocks before returning the material. As a spin-off to this work, we linked all the breast cancer cases in the HFHS tumor registry with the Detroit SEER registry to obtain survival data. We are in the process of analyzing our results, with a focus on explaining the difference in survival between African American and Caucasian women.

3.0 Specific Aim 3: Development of a Risk Factor Questionnaire

3.1 Development of Sex Hormone Exposure Index

Numerous breast cancer risk factor studies have been conducted examining various characteristics that are surrogate measures of exposure to estrogen. However, in the past, selected characteristics were often analyzed in a univariate fashion, or controlling for only a few other estrogen-related variables. Further, the number of subjects required in a study to achieve optimal statistical power becomes daunting as the number of independent variables in an analysis increases and are used in a categorical fashion. We are developing a questionnaire, using a calendar approach as a memory prompt, to inquire extensively about factors that are associated with sex hormone exposure. We are also continuing to review the literature to obtain up-to-date information on data regarding physiologic levels of estrogen and progesterone related to reproductive characteristics and exogenous hormone exposures in order to derive weights for these characteristics. Drs. Johnson, Abrams, and E. Wolman are working to integrate estimates of exposure related to various reproductive characteristics, allowing us to classify women in a relative sense as to their lifetime exposure to estrogen, as well as progesterone, in order to reduce the number of independent variables in a risk factor analysis. (Our endocrinologist, Dr. Henry Bone, has left the institution but remains in the area and is willing to continue work on the project.) Using this method, we will be able to relate cumulative hormonal exposure to various points of time in a women's life in order to examine whether cumulative exposure relative to age is important. There is reason to believe that the breast is most susceptible to carcinogenic influences at younger ages; DNA synthesis is higher in young individuals, and women under age 20 were at highest risk for radiation-induced breast cancer after atomic bomb exposure.

3.11 Variables to be Collected

We will include on the data collection instrument questions about age at menarche, lifetime menstrual cycle pattern, menopausal history, dates and duration of pregnancies, duration of lactation, infertility, history of use of oral contraceptives, fertility drugs, estrogen replacement therapy, and height and weight history.

3.12 Development of Exposure Indices

Since we will not have actual hormone exposure data for individuals in potential retrospective studies (i.e. blood levels over time), our exposure assessment will focus on the surrogate measures for estrogen and progesterone exposure listed in the survey instrument and calendar. We hope to eventually assign estimated quantitative hormone exposure scores for different reproductive characteristics during various segments of a woman's life (for example, none/low, medium, and high categories) by relying on data in the literature and on the expertise and experience of the investigators. We will explore methods to adjust, taking calendar time and age into account, for other factors collected on the questionnaire, such as parity, and apparent infertility.

3.2 Design of a Risk Factor Questionnaire Sensitive to a Multi-Ethnic Population

Focus groups, which allow for group interaction and greater insight into the meaning of certain questions in specific populations, may be used to plan and design questionnaire items or to evaluate existing ones. Discussions during focus groups are a qualitative approach to learning about psychological and sociocultural characteristics and processes in subgroups of the general population. Focus groups are typically composed of 7 to 10 participants who are usually homogenous in such characteristics as age, gender, race/ethnicity, and social characteristics.

In this study, focus groups will be used for two purposes: to develop questions that are culturally tailored to African American women in the two age groups, and to examine the perceptions of the women toward components of existing questionnaires assessing estrogen exposure and other breast cancer risk factors. These perceptions will be used to adapt existing questionnaires that have been found to be valid and reliable in the general population, to make them better suited for use among African American women. To this end, a four-hour focus group will be held for African American women under 50 years and for another group over 50 years of age. In the first two hours of each focus group session, the women will be asked to develop questions related to estrogen exposure based on overall concepts we introduce. During the last two hours of each session, the women's opinions regarding the cultural sensitivity of existing questionnaire items related to estrogen risk factors will be solicited. Specifically, the women will assess the appropriateness of the language and phrasing of the questions for African American women. Based on the comments the women generate during the focus group meetings, these existing questionnaires will be revised. Each focus group will consist of 10 members. The focus group sessions will be organized and run by trained professionals at a local firm. Each session will be videotaped for follow-up review and analysis. Focus group discussions will provide valuable insight into questionnaire design and its adaptation for African American women.

3.3 Testing of RFQ

As a part of questionnaire development, we will make it a standard practice to pilot different versions of the instrument on both African American and Caucasian women, as well as include women who vary by age and socioeconomic status. We will ask patients coming in for check-ups to HFHS gynecologists to participate as needed. Using HFHS databases to identify those women scheduled for appointments, information on age, race, insurance status and address will be reviewed. Addresses will be linked to census blocks, and together with insurance category, used to select women of varying

socioeconomic status. Self-administered and interviewer administered versions (phone and in person) will initially be constructed in the first year of the study. We will try all three to assess the feasibility of administration. The penultimate version of each questionnaire will be administered to a minimum of 10 women in the second year of the study to identify any lingering difficulties in question format. Following the development of this final draft, the following evaluations will be done:

If all three methods (self-administered, phone and in person interview) are determined to be feasible, we will test the reliability of the different methods against each other. Since these methods are, in ascending order, considerably more expensive to utilize, and because it is likely that more than one method would be useful in an etiologic study (e.g. self-administered, but phone interview for women who prefer this method) we would like to evaluate these different possibilities. We will do this by administering the questionnaire using combinations of two different methods to the same woman with a four month interval between administrations.

As a reliability test of the instrument, each type of questionnaire (self, phone and in-person) will be piloted on a new group of women and re-administered by the same interviewer 4 months after the initial interview. We hope that the intervening four months would be a long enough period to preclude retained memory of previous responses to the questionnaire. Variables that are not time-sensitive will be analyzed for comparability, taking into consideration changes that may have occurred over 4 months.

Finally, we will assess intra-interviewer reliability for the interviewer-administered versions of the instruments determined to be feasible and have inter-interviewer reliability. Two different interviewers will administer, using the same methodology, the identical questionnaire to the same subject 4 months apart. Again, comparisons will be made between non-time-sensitive variables.

Each of the reliability assessments will be made in the subgroups of Caucasian women and African American women, as well as pre-menopausal and post-menopausal women.

3.4 Sample Size and Analysis Plan for RFQ Testing

Although we are concerned about the reliability of the entire questionnaire, we are particularly concerned about those items that relate to estrogen exposure. For the evaluation of the reliability of continuous variables such as age at menarche, duration of lactation, age at menopause, and height and weight history collected on different instruments, we will use a balanced incomplete design, since each participant will be given two of the three possible types of questionnaire administration; self-administered, telephone interview or in-person interview. We estimated the power of the tests using paired t-tests. We plan to administer each pair of interview-administration combination to 65 women, e.g. 65 will receive self-administered and telephone interview, 65 will receive telephone and in-person interview, and 65 will receive in-person and self-administered interview. With those numbers we will have 90% power to test effect sizes of 0.5 using a type I error of 0.01 to account for multiple comparisons. McNemar's test will be used to compare binomial responses such as ever used contraceptive drugs, ever used fertility drugs, etc.

3.5 Use of RFQ Results

Based on the results from the reliability studies, a finalized version of the questionnaire(s) will be completed. Recommendations will be made as to whether different data collection modalities may be employed in future studies using these instruments.

Progress for Aim 3

The risk factor data collection tool, the Women's Health Questionnaire, is currently under development. We have collected and reviewed questionnaires from the major breast cancer epidemiologic studies across the country. Our goal is to develop a questionnaire that includes data characterizing demographic, social, dietary and lifestyle factors, endogenous and exogenous hormonal exposure, reproductive history and mammography outcomes.

exposure, reproductive history and mammography outcomes.

We have selected one, that used by the Women's CARE study, as the basis for our instrument. We are in the process of converting the CARE study questionnaire, which is administered in a personal interview, to a telephone administered questionnaire and a self-administered questionnaire. As soon as it is completed, we will pilot the three versions of the questionnaires, and review them for cultural sensitivity using focus groups. Early in January through May we will conduct reliability studies comparing the different questionnaire versions administered to groups of women.

We have met several times with Dr. Eric Wolman, and reviewed the medical literature related to modeling estrogen exposure and breast cancer risk. One paper has been published that included some of the results of Dr. E. Wolman's review (see Appendix). He is in the process of ascertaining constants to be utilized in our final model.

Conclusions

Progress has been as planned, with the exception that the pathology review is taking longer than anticipated.

To address this study's Specific Aims, we plan to accomplish the following tasks within the next (and final) funding year:

-Complete the establishment of the BBD cohort

-Continue the microscopic review of BBD cohort pathology slides

-Complete the storage and documentation of pathology material

-Complete the cohort data base

-Finalize the Risk Factor Questionnaire instruments

-Test the Risk Factor Questionnaire instruments

-Write up our results for publication

Our results should yield a well-documented cohort and data base from which to generate study ideas. We should also have several versions of a risk factor questionnaire to be used in studies evaluating reproductive and medication related variables in women's health studies, especially epidemiologic studies of breast cancer.

Appendix

New directions in breast cancer research[1]

SANDRA R. WOLMAN,*,[2] GLORIA H. HEPPNER,† AND ERIC WOLMAN‡

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ABSTRACT **Research in breast cancer extends in many directions, stimulated by concerns related to the high incidence of the disease and the relative unpredictability of its clinical course. Examples of work in several directions are presented here arranged by four levels of analysis. *1*) Molecular, intracellular events (molecular genetics). Recent identification of genes that predispose to breast cancer, and the isolation of those genes and their protein products, permit investigations of the most critical issues: the roles of these genes in normal development and breast differentiation, and how their alteration permits or contributes to tumor initiation. Thus, we expect that understanding the functions of the genes involved in inherited susceptibility to breast cancer will also be informative for sporadic breast cancers. *2*) Cellular biology (cellular models for preneoplastic disease). We examine models of breast cancer development and ask how they help to validate a morphologic sequence for human breast neoplasia and whether they permit investigation of how to modify disease progression. Two useful models, one in transgenic mice and the other using human breast stem cells capable of culture and xenograft growth, are now available. *3*) Tissue and organ (the tumor and its local environment). We look at the relationship of the tumor cell population to its local environment (stroma, blood vessels, etc.). This leads naturally to questions of how neighboring tissues and cytokines may modify tumor growth. *4*) The individual as an organism and member of a population (hormonal risk and chemoprevention). We address identification of the primarily hormonal risk factors and a possible related mode of cancer prevention.—Wolman, S. R., Heppner, G. H., Wolman, E. New directions in breast cancer research. *FASEB J.* 11, 535–543 (1997)**

Key Words: molecular genetics · cellular models for preneoplastic disease · tumor environment · hormonal risk · chemoprevention

MOLECULAR GENETICS

THE RECOGNITION THAT ALTERATIONS of *BRCA1*, more recently *BRCA2*, and potentially a few other genes are responsible for increases in risk of breast cancer incidence on a familial basis has led to a host of previously unapproachable research questions and clinical opportunities. The *BRCA1* gene was identified by linkage studies (1) that permitted a focus on localized chromosomal regions, and eventually led to cloning and sequencing of the gene (2). The normal function of *BRCA1* is unknown, but it appears to confer tumor suppressor activity. Some clearly familial but *BRCA1*-negative cases were recognized; a second gene, *BRCA2*, was identified rapidly by similar approaches (3). The classic breast cancer families show multigenerational tumors and are associated with tumors of relatively early onset, often bilateral or with multiple primary sites. These hereditary cancers appear to display an altered histologic spectrum and outcomes that differ from those suggested by other prognostic indicators; it has been reported that *BRCA1*-related cancers are associated with lower recurrence and death rates than would be expected for their aneuploidy and high proliferative activity (4). Other tumors often segregate within the same families: ovarian cancers with *BRCA1*; *BRCA2* is associated with male breast cancers, pancreatic cancers, and possibly others.

BRCA1 and *BRCA2* account for most of the families with high and multigenerational breast cancer incidence, with little evidence that a third or fourth gene is responsible for substantial numbers of cases. However, other known genes contribute to inherited risk of breast cancer, notably the p53 gene in Li-Fraumeni syndrome, a locus on 10q in Cowden syndrome, the *ATM* gene at 11q22–3 in ataxia telangiectasia, and probably the dominant gene in the breast and colon

[1] The topics included here were discussed in an American Society for Investigative Pathology symposium, "New Directions in Breast Cancer Research," presented on June 5, 1996 at the Joint Meeting in New Orleans, Louisiana. We thank the speakers at that symposium, Steven Narod, Fred Miller, Judah Folkman, and Malcolm Pike, for their major contributions to this review. It is intended to illustrate by example, rather than to review comprehensively, some exciting new developments.

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cancer families described by Lynch. At least one of these, *ATM*, may contribute considerably to the risk of apparently "sporadic" breast cancer in the heterozygous carrier (5).

BRCA1

Women from families with altered *BRCA1* show a peak breast cancer incidence between ages 45 and 50, with a later peak for ovarian cancer. In these families the lifetime risk of breast cancer for the gene carrier is more than 85%; in some families that for ovarian cancer is more than 70% (6). Knudson's two-mutation model suggests that a second mutation occurring, on the average, in adolescence with a latent period for tumor development could account for the early age peak instead of the more usual continuing increase of cancer with age. As is true for sporadic breast cancer, the risk of breast cancer in gene carriers is reduced by increased parity, but unfortunately the risk of ovarian cancer is thereby increased. The ratio of the two tumor types varies among the affected families.

BRCA2

In families with *BRCA2* gene damage, the peak tumor age is even younger than for *BRCA1* (4), and there is a clear association with male breast cancer. In fact, in Iceland 40% of males with breast cancer appear to be members of *BRCA2* mutant families. More recently, associations with pancreatic cancer and the rare cancer of Fallopian tubes have been established in some *BRCA2* families. Ovarian cancer is less often associated with *BRCA2* than with *BRCA1*. A connection with prostatic and colon cancer is also suspected. However, detection of *BRCA2* mutations has been far more difficult than for *BRCA1* (7), which has hampered efforts to delineate the spectrum of associations more fully. In addition, the penetrance (the degree to which the mutation results in expression of the disease phenotype) of the different mutations is unknown.

Mutation detection and screening

Mutations appear widely distributed throughout the large *BRCA1* gene. (Approximately 100 kb overall, it has 22 coding and 2 noncoding exons, and the transcript is 7.8 kb in length.) Many different mutations have been identified and a few are relatively common. The mutation frequency appears slightly lower at the 3′ end of the gene. Deletions (adenine-guanine) are most common and often result in a truncated protein product (the normal 200 kDa protein contains 1863 amino acids), with insertions (cytosine) next in frequency. At least one mutational cluster has been identified; it is a specific recurring deletion in the *BRCA1* gene at position 185 that has been found in an ethnic cluster of Ashkenazi Jews. Although this could represent a mutational "hotspot," it is more likely that these families share a common origin.

The previously reported increased frequency of rare alleles of the *RAS* oncogene, which did not hold up as a risk factor for breast cancer (8), has been noted in more than half the ovarian cancer patients within *BRCA1* families (9). There may also be substantial differences in intrafamilial penetrance of the phenotype. It now appears that ovarian cancer predominates in Pakistani families, whereas breast cancer is the main lesion in Guatemalan families. Loss of heterozygosity at the *BRCA1* locus has been identified in nearly half of sporadic breast carcinoma cases as well as in the familial disorder (10).

Mutations in *BRCA2*, which is nearly twice as large as *BRCA1*, have been more difficult to pinpoint; in fact, mutations (mainly small deletions) were identified in only 8 of 49 families with site-specific breast cancer and without ovarian cancer (7). In many families where the inheritance pattern is clear and the clinical manifestations are characteristic, the genic alteration may reside in a mutation outside the coding region or may be dependent on a second gene.

Two critical issues are raised by the discovery of *BRCA1* and -2. First, because these genes are large and complex, new techniques to detect their mutations are required. Although lesions of *BRCA1* or *BRCA2* may be detected by linkage, by gene sequencing, or by detection of a truncated protein, present methods are not sufficiently sensitive to detect more than two-thirds of cases. In addition, sequencing is slow and cumbersome. Screening is clearly feasible in some Jewish families because of specificity of the mutation site. However, if the different mutations result in varying degrees of disease penetrance, the individual's risk of breast cancer development will also vary by family. The nature of patient concerns makes better definition of both clinical and molecular means to assign risk to the individual highly desirable. The second issue is that the products of these genes may be lost or otherwise altered in sporadic breast cancer, so that understanding their roles in the inherited case should illumine both forms of the disease.

MODELS FOR PRENEOPLASTIC BREAST DISEASE

One of the most influential concepts in modern cancer biology is that developed by Vogelstein and associates, which relates specific alterations in cancer-related genes to a sequence of morphological events leading to the development of colon cancer. This concept could apply, in principle, to the development of other types of cancer, including breast

cancer. Unfortunately, the identification of either morphological or molecular precursors of breast cancer is not nearly as clear as it is in colon cancer. Whether differences between the two organ sites are due to fundamental differences in biology or to logistical differences in early disease detection (11), the need for experimental approaches to understanding preclinical events in breast cancer is evident.

Murine models of preneoplastic breast disease

The prototypic model of mammary gland preneoplasia is the hyperplastic alveolar nodule (HAN)[3] of the mouse, a focal lesion observed in mice at high risk for developing mammary cancer. HANs are considered "preneoplastic," because when transplanted into gland-free mammary fatpads of syngeneic mice, the hyperplastic outgrowths frequently develop focal mammary tumors, unlike transplants of normal ducts (12). HAN lines developed by Medina (13) have proved valuable in elucidating the roles of viruses, chemical carcinogens, hormones, growth factors, etc., in the progression from HAN to tumor. Although the relevance of the HAN model to high-risk lesions in humans (see below) is not direct, it illustrates basic principles of neoplastic progression in the mammary gland and can be used to test hypotheses relating the role of specific oncologic events to mammary cancer progression, as well as strategies for prevention.

More recently, transgenic models of murine hyperplasia and cancer have come to prominence (14). Transgenic models are designed to test the ability of a specific gene (oncogene, suppressor gene) or combination of genes to drive cancer development. Genes have been chosen for study on the basis of their relevance to human cancers (*RAS, ERBB2, C-MYC, TP53*), mouse mammary cancers (int-2), other animal models (polyoma middle T, SV40 large T), or all three. The mammary hyperplasias and cancers seen in these models do not clearly reflect the natural history of human breast cancer development. Thus, these models show that a gene (or genes) *can* effect mammary tumor development, not whether it *does* so under "field conditions." The models are highly variable, as are the tumors they generate, depending on the particular gene under study, the promoter used to express it, and the degree of tissue specificity achieved.

Cardiff (15) has systematically described the histopathology of more than 750 cancers and hyperplasias from a variety of different transgenic constructs. He found that the morphology of these lesions is a reproducible characteristic of the specific transgene, suggesting that particular transgenic models may be analogs of cancer development pathways in subsets of women, for example, having the unusual breast cancer morphology observed in *BRCA1* and *-2* carriers (4). The focal atypias (rarely HANs) observed in transgenic mammary glands imply that secondary genetic changes, not the transgene alone, are involved in neoplastic development.

Preneoplastic breast disease in women

A key element in the control and prevention of invasive breast cancer is the recognition of early, preclinical changes that identify a woman as being at high risk to develop the disease. The histological abnormalities described by Page and associates (16), and collectively known as proliferative breast disease (PBD), are associated with increased risk of developing breast cancer; the lesions and respective risks range from moderate hyperplasia (twofold risk by 15 years) to atypical hyperplasia (fourfold) and carcinoma-in-situ (CIS) (11-fold). The biological relationship of these high-risk lesions to each other and to clinical carcinoma is uncertain. An experimental model of PBD is needed to test whether the sequences of molecular events underlying the development of breast cancer are indeed analogous to those represented by Vogelstein's model of colon cancer.

An ideal model of the ways that PBD leads to breast cancer would have the following features: *1*) it would faithfully reproduce the histological spectrum, from moderate and atypical hyperplasia to CIS, while also displaying normal ductal morphology; *2*) it would mimic the heterogeneity of PBD with any or all of these lesions, including carcinoma, present in a single patient; *3*) it would lead to invasive malignancy with some frequency; *4*) its time course would allow for the highly sporadic nature of human cancer development; and *5*) it would mimic the principal cellular and molecular events that occur in human PBD.

The MCF-10AT model

Although not ideal, a model of PBD that meets many of these criteria is the "MCF-10AT" family of cell lines developed by investigators at the Karmanos Cancer Institute (formerly the Michigan Cancer Foundation) (17). Its origin is the MCF-10A line developed by Soule (18) from breast tissue of a woman with mild hyperplasia. MCF-10A is one of a series of lines established through spontaneous immortalization of the original, mortal cultures. MCF-

[3] Abbreviations: HAN, hyperplastic alveolar nodule; PBD, proliferative breast disease; CIS, carcinoma in situ; ER, estrogen receptor; ERT, estrogen replacement therapy; IGFs, insulin-like growth factors; VEGF, vascular endothelial growth factor ; bFGF, basic fibroblast growth factor; AI, angiogenesis inhibitor; RRs, relative risks; E2, estradiol; Pg, progesterone; SHBG, sex hormone binding globulin; GnRHA, gonadotropin-releasing hormone agonist; FFTP, first full-term pregnancy; BMD, bone mineral density.

10A cells were transfected with mutated T24 H-ras, yielding a line called MCF-10AneoT. Unlike MCF-10A cells, MCF-10AneoT cells, suspended in Matrigel, persist as xenografts in nude or nude/beige mice. About 30% of the grafts develop carcinoma at times ranging from 50 days to 2 years after injection. If recultured and injected into new nude/beige hosts, these xenografts do not grow out initially as carcinoma, but instead produce histologically complex structures—some with normal breast duct morphology, others with lesions of PBD and invasive cancer (**Fig 1**).

Serial reculturing of xenografts and reinjecting the cells from invasive cancers as well as hyperplastic grafts into nude/beige mice have generated the family of MCF-10AT lines. Never has any of these lines grown out directly as a carcinoma after injection into nude/beige mice; they have all reverted to normal or hyperplastic structures. With ascending serial passages, the onset of PBD has appeared earlier and the incidence of lesions is greater; development of invasive cancers has remained at about 20–25% and continues to be sporadic. The entire morphologic range—normal ducts (including both epithelial and myoepithelial cells), PBD, CIS, and cancer—indicates that MCF-10AT is a developmental and neoplastic stem-cell line (19); the same spectrum also develops from clonal variants.

The MCF-10AT family appears, histologically and behaviorally, to be an appropriate model of human PBD. It reinforces the hypothesis that the lesions comprising PBD are related directly to the development of human breast cancer. Studies at the biological and molecular levels are in progress. The model expresses functional estrogen receptor (ER) even though the parental MCF-10A line is ER-negative (20). MCF-10A and MCF-10AT cells express the same levels of wild-type p53 protein, although they express a conformationally altered (not point-mutated) p53 as well. The altered form of p53, which increases with increasing passage generations of MCF-10AT, is able to block wild-type p53 function in in vitro assays, suggesting that it may be a determinant of progression in this system (21). Other features of breast cancer development are likewise mimicked in the model. For example, the proliferation index increases from hyperplasia to atypical hyperplasia to CIS to cancer, as does the expression of the *ERBB2* oncogene (22). Reflecting the variability of human disease, the carcinomas present a broad histologic spectrum, with some showing glandular differentiation, others a squamous pattern, some mixed, and some undifferentiated. Based on immunohistochemistry, the development of adenocarcinoma appears to be part of a continuum from hyperplasia to atypical hyperplasia to CIS to cancer, whereas the development of squamous cancers is not (22).

A feature of the MCF-10AT model that does not appear to mimic human PBD or breast cancer is the presence of mutated *RAS*. *RAS* mutation is rare in human breast disease, although overexpressed ras is not (23). Its significance in the model is unclear. Clones of MCF-10AneoT that fail to produce lesions in nude/beige mice nevertheless have the same level of mutated ras, at the same insertion site, as do the lesion-producing MCF-10AT lines (24). A majority of breast tumor lines overexpress the ras-related protein TC21, and aberrant function or overexpression of TC21 in MCF-10A cells results in in vitro transformation (23). Thus, alterations in ras signal transduction pathways may be important to human breast cancer development even though point mutation is not the usual mechanism of alteration. MCF-10AT seems to satisfy at least four of the five criteria given above for a model of PBD.

THE TUMOR AND ITS LOCAL ENVIRONMENT

Stromal interactions

A recurrent, but often overlooked, theme in breast cancer research is the recognition that "the cancer cells"—that is, the tumorous epithelial cell compartment—are not equivalent to "the cancer." Rather, breast cancers are tissues that contain numerous other types of cells (normal epithelial cells, myoepithelial cells, fibroblasts, myofibroblasts, endothelial cells, smooth muscle cells, inflammatory cells) and acellular components of basement membrane and extracellular matrix. These are not independent but interactive. The concept of mutually interactive cellular and acellular components, termed "dynamic reciprocity" by Bissell (25), is a feature of the developing, the reproductively mature, the postmenopausal, and the neoplastic breast. An exciting focus of current research is on the molecular mechanisms underlying the dynamic reciprocity of epithelium and stroma within breast cancers. For example, insulin-like growth factors (IGFs) are mitogens for normal and neoplastic breast epithelium. IGF-I mRNA is expressed in normal breast tissue fibroblasts and, to a lesser extent, in fibroblasts from breast cancers. IGF-II mRNA, however, not only is found at low levels in normal fibroblasts but also is a predominant feature of cancer-associated fibroblasts. Coculture of normal breast fibroblasts with MCF-7 breast cancer cells leads to an increase in IGF-II mRNA in the fibroblasts, suggesting that breast cancer cells are able to elicit cooperation from stromal cells in the form of paracrine growth factors, thereby furthering their own proliferation (26).

Another example of stromal-epithelial interactions in breast cancer relates to the mechanisms of tumor

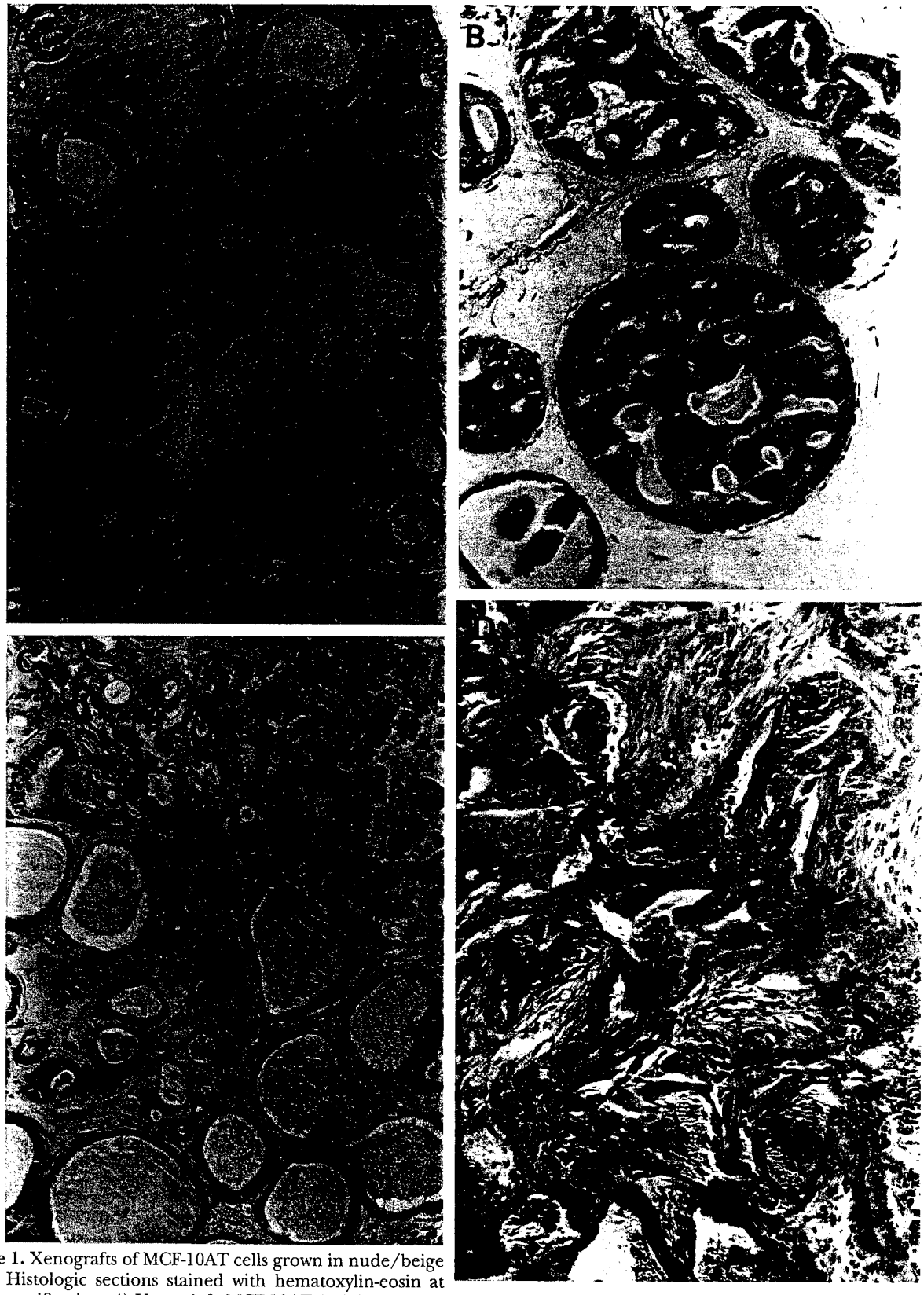

Figure 1. Xenografts of MCF-10AT cells grown in nude/beige mice. Histologic sections stained with hematoxylin-eosin at ×25 magnification. *A*) Upper left: MCF-10AT (original inoculum), lesion showing mild hyperplasia with dilated duct and layered epithelial lining; resected on day 223 after transplant. *B*) Upper right: MCF-10AT3C (third transplant generation), hyperplasia with cribriform intraductal proliferation and atypia; day 166 outgrowth. *C*) Lower left: MCF-10AT3B (third transplant generation), benign ducts are present in the lower half of the figure, with invasive carcinoma above; day 116 lesion. *D*) Lower right: MCF-10AT (original inoculum), showing a small, moderately differentiated adenocarcinoma that was detected 393 days after transplant.

invasiveness. Cancer cells secrete a variety of proteases that apparently aid in breaking down extracellular matrix components and facilitating invasion. However, cancer cells are not the only contributors to this process. A variety of stromal cells, including fibroblasts associated with the invasive edge of the cancer, are also sources of invasion-facilitating proteases, including stromelysin-3 and urokinase plasminogen activator (27). Again, cooperation between cancer cells and stromal elements leads to furtherance of the cancer's agenda.

The importance of the acellular components in dynamic reciprocity is illustrated by the fact that basement membranes are able to bind, sequester, and inhibit a variety of different cytokines (27). Proteolytic cleavage of basement membrane components, by either cancer or stromal cell proteases, results in the liberation of these factors in active forms.

Understanding the role of stromal cells and other host components in breast cancer development and progression is an important cornerstone of basic breast cancer biology. Its potential for approaches to prevention and therapy is less certain because the principles and processes involved may not be amenable to selective interference. Further, such interactive systems are often characterized by redundant mechanisms that keep the whole functioning even when a particular pathway is crippled, i.e., the process of carcinogenesis is itself "homeostatic." There is, however, one component of the tumor microenvironment that does appear to be a susceptible target for intervention: the tumor vasculature.

Angiogenesis

Both primary and metastatic tumors must be able to induce blood vessel formation in order to grow. Angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) permit the growth of tumors by eliciting new blood vessel formation. Without such factors the tumors would remain small, clinically undetectable, and irrelevant to host survival.

The concept of tumor dormancy was developed to account for the sudden reappearance of clinical disease after the presumably successful removal of a primary lesion. Dormancy is most characteristic of tumors that show recurrence or metastasis years later. They presumably grow from microscopic, nonexpanding populations of tumor cells. A related phenomenon is the often observed sudden growth of metastases upon removal of a primary tumor. In the absence of new blood vessel formation there is suppression of distant metastases, and the microscopic tumor foci demonstrate increased incidence of apoptosis (28). Thus, the capacity for dormancy and metastatic suppression by the primary lesion suggest that tumors may elaborate angiogenesis inhibitors as well as angiogenesis factors.

Several anti-angiogenesis factors have been detected. The first tangible evidence of secretion of an angiogenesis inhibitor (AI) by a primary tumor was derived from studies of the murine Lewis lung carcinoma; the inhibitor, called angiostatin, appears to inhibit endothelial cell proliferation specifically and is a potent inhibitor of metastatic growth in this experimental system (29). Its action is opposed to the VEGF described by Dvorak (30), which is also secreted by the primary tumor. However, the longer half-life of angiostatin in the circulation results in the suppression of distant metastases as the primary lesion expands. Angiostatin copurifies with plasminogen and is activated upon release, but the mechanisms by which it is activated and released are not yet understood. A corresponding purified fragment of human plasminogen appears to have AI activity in the same experimental mouse model, whereas intact plasminogen permits extensive vascularization of tumor and metastatic growth. A model system to test for generation of AI activity by other primary tumors has been developed and entails the growth of tumors in SCID mice, with implantation of pelleted bFGF in the cornea of the animal host to test for circulating AI (31). A new AI called endistatin has been recognized recently that has structural homology to the carboxyl terminus of collagen XVIII.

The remarkable direct visualization system developed by Chambers (32) has permitted observations of the extravasation and migration of potentially metastatic cells. These observations suggest that interaction with extracellular matrix is critical to metastatic capability, that there are preferred vascular-rich sites within target organs, and that autocrine and paracrine effects may be essential to further growth. Interleukin 6 has proved to be a potent motility factor for breast cancer cells, and it is clear that tumor cells have receptors for such motility factors.

Anti-angiogenic factors may become important therapeutic agents (and some anti-cancer drugs such as Taxol have demonstrated AI activity). Experimental use of angiostatin in tumor-bearing mice resulted in regression of tumor without host toxicity; in the residual dormant tumor foci, cell proliferation was balanced by apoptosis (33). If the primary lesion were removed, AI agents could induce and extend dormancy, and there is always the possibility that, during the dormant phase, other interventions could lead to complete clearance of the tumor cell population. Clearly, one important investigative direction is toward chemotherapeutic agents that are taken up by or can metabolically alter endothelial cells.

HORMONAL RISK AND CHEMOPREVENTION

Epidemiologic evidence

When they were first identified, most of the recognized risk factors for breast cancer seemed to confer relative risks (RRs) typically less than 2, in contrast, for example, to the enormous RR of malignant mesothelioma from exposure to asbestos. When an RR exceeds unity by only a few tens of percent, the causative factor and its mechanisms of action are hard to identify, and preventive measures hard to validate, because of the huge sample sizes needed in epidemiological studies and clinical trials. However, it was proposed in the 1960s that several apparently distinct risk factors for breast cancer (especially early age at menarche and late menopause) could be viewed as aspects of a single factor—viz., total exposure to estrogen and progesterone—with a much greater influence on susceptibility to the disease (34–36).

A necessary step in the chain of causality leading to breast cancer is the proliferation of breast cells. The risk of breast cancer increases rapidly from menarche to menopause (either natural or surgical), when breast epithelial cell division rates, particularly in the terminal-duct lobular units, are high during the luteal phase of each menstrual cycle. Risk continues to increase after menopause, but much more slowly; during this time the cyclic production of estradiol (E2) and progesterone (Pg) in large quantities is replaced by constant levels of E2 (low) and Pg (extremely low) (34).

Even though E2 is a breast cell mitogen, the importance of Pg is suggested by the fact that breast epithelium proliferates very slowly in the follicular phase, when E2 reaches its highest level but Pg is nearly absent (35). The use of postmenopausal estrogen replacement therapy (ERT) for more than 10 years may well raise breast cancer risk by at most several tens of percent; estrogen-progestin hormone replacement therapy may raise the risk a little more than estrogens alone, but not by an amount that has been shown to be statistically significant (37–39). [This consistent picture, involving both endogenous and exogenous hormones and not yet fully confirmed, took many years to emerge, in part because Pg clearly opposes the role of estrogen in endometrial carcinogenesis (34, 35).]

The hormones used in combination-type oral contraceptives, whose formulations and dosages have evolved substantially to avoid increasing breast cancer risk, differ from the endogenous hormones, and the timing of their concentrations in the natural and pill-regulated cycles differs considerably. However, total breast cell proliferation seems quite similar in the natural and contraceptive cases; so does the rate of increase of breast cancer risk with time, at least for cancers diagnosed postmenopausally (35, 40).

Obesity after menopause causes additional risk by raising the levels of estrogens and free E2 [i.e., not bound by sex hormone binding globulin (SHBG)], because estrogens are produced in adipose tissue and obesity lowers SHBG levels. Obesity is somewhat protective in women under age 40, giving rise to anovulatory cycles and also lowering luteal-phase Pg after ovulation (35, 41). Correspondingly, longer (and so less frequent) and irregular menstrual cycles are protective, but this observation is difficult to quantify because of the need for precise recall or records (34).

Estrogen and Pg levels become very high during pregnancy. A first full-term pregnancy (FFTP) thus adds to breast cancer risk, but also causes differentiation of breast epithelium that leaves it less vulnerable to genetic damage (34, 36, 42). An early FFTP elevates the immediate risk in young women, but its protective effect dominates after age 40. If, on the other hand, the FFTP occurs late enough—certainly after age 35—the protective effect seems to be lost, resulting in a risk of breast cancer higher than that of nulliparous women (35, 36, 43).

Chemoprevention

These and similar epidemiological observations suggesting a central role for E2 and Pg in breast cancer have led to a testable strategy for primary chemoprevention. Ovulation and the production of ovarian steroids can be completely suppressed by the continual administration of a gonadotropin-releasing hormone agonist (GnRHA). This by itself would cause hot flashes, vaginal dryness and bleeding, loss of bone mineral density (BMD), and other side effects; these hypoestrogenic symptoms can be prevented by ERT near the low level now used postmenopausally, with a progestin given every fourth cycle to protect against endometrial hyperplasia. To prevent BMD loss, just enough androgen must be added to make up for the loss of free testosterone caused by the GnRHA. This experimental regimen is intended to reduce the risk of breast cancer and to protect against cardiovascular risk, ovarian and endometrial cancer, and BMD loss. A small pilot trial used the GnRHA leuprolide acetate (44). HDL cholesterol rose in the trial; symptoms of premenstrual syndrome were also ameliorated. Putative evidence of the central benefit was that the mammographic densities of the trial women were sharply reduced, which was interpreted as a sign of reduction in mitotic activity and hence in the risk of cancer.

Current issues

This experimental approach, with its large predicted reduction of breast cancer risk, continues to be tested and refined. The epidemiological argument sketched above is not universally accepted and needs

strengthening. There is no doubt that estrogens and progestins are key factors in breast cancer risk; but although observations confirming the importance of total exposure continue to accumulate, so do some conflicting data and interpretations, suggesting that the individual reproductive risk factors may also have separate influences (36, 38, 40, 41, 45).

Four interrelated issues bear on the validity and completeness of the total-exposure approach. The most fundamental questions pertain to the molecular bases of the hormonal influences on normal and carcinogenetic pathways. The arguments given for the proposed regimen emphasize the proliferative role of the ovarian hormones (35, 44), but any possible contribution of direct genotoxicity must also be understood.

Although the simple hypothesis concentrating on estrogens and Pg has considerable explanatory power, it is known that breast tissue interacts in relevant ways with androgens, prolactin, insulin, insulin-like growth factors, epidermal growth factor, transforming growth factor β1, and probably other hormones and growth factors (35, 41, 44, 46). The recent identification of BMD (which is known to increase with increasing levels of endogenous estrogens) as a direct indicator of breast cancer risk in women over age 65 (after adjusting for 12 other known risk factors, six of them directly hormone-related) illustrates the complexity of the problem (46).

The third issue is the role of the "lifestyle" factors: diet and exercise. Dietary fat as a risk factor has been studied in Europe and America for many years, inconclusively for some time because of the inherent difficulty of dietary recall, the apparently modest influence of this factor, and its dependence (not widely recognized until the 1980s) on the particular fats consumed. Yet one of the most striking observations of cancer epidemiology is the wide variation in breast cancer risk among countries. This is a disease of high socioeconomic status, at least in part because of fat consumption; it is prevalent in North America, Scandinavia, and northern Europe, but much rarer in Asia. The age-adjusted incidence in the U.S. is roughly fivefold that in Japan (35, 43). When Asian families migrate to the U.S., their risk of female breast cancer rises over several generations toward "American" levels (41). Although the within-culture variability of risk is moderate, it is very large among cultures.

The hormonal hypothesis offers a tentative explanation of this puzzling situation. Age at menarche is a consequence of energy balance (diet and exercise) in the years leading up to it and occurs on the average 2 years later in Japan than in America. Late menarche is associated with even later establishment of regular menstrual cycles, adding to its protective effect. Postmenopausal Japanese women are much slimmer than their American counterparts; their estrogen levels are correspondingly much lower and their risk of breast cancer hardly increases at all after menopause. Premenopausal Asian women with traditional lifestyles have substantially lower E2 levels than typical American women, and the average menstrual cycle in Japan is more than 6% longer than that in the U.S. In the light of the mathematical model discussed below, these observations are sufficient to account quantitatively for the intercultural difference in risk (35, 43). The resulting picture is consistent with the extremes of low risk found in America—with vegetarianism and athletic amenorrhea, for example.

This argument suggests that diet and exercise are, like the timing of menarche, FFTP, and menopause, really aspects of the same fundamental risk factor: total exposure to estrogens and Pg. (Exercise seems to have its principal influence through its effect on adiposity.)

The fourth issue is the role of Pike's mathematical model of cumulative exposure to ovarian hormones and of cumulative cell division in understanding the epidemiology of breast cancer. For many non-hormone-dependent cancers, the incidence I is proportional to the age t raised to the power k. For breast cancer, $I(t)$ has the same form if t is replaced by a function $d(t)$ representing cumulative *d*uration of exposure to the relevant insult, and k is 4.5. The time rate of change of $d(t)$ can be thought of as an "effective mitotic rate" or "intensity of exposure," which is zero before menarche, rises quickly to 1 with the establishment of regular cycles, has an upward jump at FFTP and immediately falls to a value of around three-quarters, remains constant into the 40s, and falls slowly around menopause to a postmenopausal value near 0.1 (43).

The processes of refining such a model and of clarifying hypotheses about reproductive risk factors play complementary roles. The accumulation of data suggests improvements to the structure and parameter values of the model, and each version of the model poses questions for theory and observation. Should a model of this type prove reasonably robust, it could simplify the analysis of observations and trials by collapsing many related variables into a single one. Pike's group (43, 44) has used the model as a guide for years, in particular to predict their experimental regimen's reduction of risk for breast cancer. Others have extended the model to account in detail for the effects of parity in the Nurses' Health Study (47).

Research at all levels, from molecular to population, should continue to be directed toward modifying, confirming, and explaining the sex-steroid exposure hypothesis. FJ

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[4] Many of the references are to summaries and reviews, which in turn cite the primary literature.

HENRY FORD HOSPITAL

2799 West Grand Boulevard
Detroit, Michigan 48202

October 7, 1997

Subject's Name
Street Address
City, State/Province Zip/Postal

Dear [*Recipient*]:

The prevention of disease is a very important part of health care. We at Henry Ford Health System are very interested in disease prevention among women. As a woman who at some time has received health care at Henry Ford Health System, we would like to invite you to be part of an exciting women's health project.

To participate in this project, one of our staff members will be calling you in the next few weeks to conduct a short health survey which should only take 10 minutes of your time. If you agree, you may be contacted in the future and asked additional questions. It is important for you to know that all information you provide will be kept confidential. There is no cost involved and the medical care that you receive will not be affected by this project.

Your participation is completely voluntary but we hope you will join us in this important research project. You can help us understand how to keep women healthy. If you have any questions about this project, please feel free to call Angela Blount at (313) 874-6232.

Sincerely,

Maria Worsham, PhD
Molecular Biologist
Women's Health Study

Christine Cole Johnson, PhD, MPH
Epidemiologist
Women's Health Study

BENIGN BREAST DISEASE STUDY

DATA SHEET

MRN ___ ___ ___ ___ ___ ___ ___ ___

Pathology Report # ___ ___ -___ ___ ___ ___ ___ Date Abstracted ___ ___ / ___ ___ / ___ ___

Date of Index Pathology Report ___ ___ / ___ ___ / ___ ___ Interviewer's Initials ___ ___ ___

	PRINTED	CORRECTED
1. Name	_____ _____ _____ (last / first / mi)	_____
2. Maiden or Other Names	_____	_____
3. Address	_____	_____
	_____	_____
4. Home Phone	(___ ___ ___) ___ ___ ___-___ ___ ___ ___	(___ ___ ___) ___ ___ ___-___ ___ ___ ___
5. Work Phone	(___ ___ ___) ___ ___ ___-___ ___ ___ ___	(___ ___ ___) ___ ___ ___-___ ___ ___ ___
6. Emerg. Phone	(___ ___ ___) ___ ___ ___-___ ___ ___ ___	(___ ___ ___) ___ ___ ___-___ ___ ___ ___
7. Social Security #	___ ___ ___-___ ___-___ ___ ___ ___	___ ___ ___-___ ___-___ ___ ___ ___
8. Marital Status	_____	_____
9. Spouse's Name	_____	_____
10. Birth Date	___ ___ / ___ ___ / ___ ___ ___ ___	___ ___ / ___ ___ / ___ ___ ___ ___
11. Sex	_____	_____
12. Race	_____	_____

[INT: DO NOT CONFIRM, GO TO LOCATOR QUESTION #8]

13. Status	_____	_____
14. Date of Death	___ ___ / ___ ___ / ___ ___ ___ ___	___ ___ / ___ ___ / ___ ___ ___ ___

BENIGN BREAST DISEASE STUDY

LOCATOR FORM

All study subjects have been mailed an introductory letter briefly explaining the study. As an interviewer, you will be calling subjects to administer a short health survey. All numbered survey questions should be read. Instructions and survey codes are enclosed in [ ].

INTRODUCTION:
"Hello may I speak with **[Subject]**? Hello, my name is **[Interviewer]** and I am calling from a women's health study being conducted by Henry Ford Health System. We recently sent a letter telling you about our study looking at the prevention of disease among women. As a woman who at some time has received medical care at Henry Ford, I would like to ask you some questions about your health. All information you provide will be strictly confidential. This will only take a few minutes."

[IF SUBJECT IS DECEASED OR UNABLE TO ANSWER THE QUESTIONS: Explain study to contact person and ask them if they will complete Locator Form questions #5 and 7 as it relates to the study subject. State that we may need to contact them for additional information about the subject. Ask the contact person for their name, address and phone number and record on the corrected side of the Locator Sheet. Record who completed the form on page 6.]

[IF SUBJECT DID NOT RECEIVE THE LETTER: Paraphrase the letter to the subject. If they would like another copy of the letter sent to them, verify their name and address and inform them you will be calling back after the letter is mailed.]

1. On average, how often do you see your primary care physician? **[Read 1-4]** _____

    1. More than once a year
    2. Once a year
    3. Once every 2-3 years
    4. Less than every 4 years
    9. Don't Know

2. On average, how often do you receive a mammogram? **[Read 1-4]** _____

    1. More than once a year
    2. Once a year
    3. Once every 2-3 years
    4. Less than every 4 years
    9. Don't Know

3. On average, how often do you have a pap smear? **[Read 1-4]** _____

1. More than once a year
2. Once a year
3. Once every 2-3 years
4. Less than every 4 years
9. Don't Know

4. Have you ever been diagnosed with ovarian cysts? **[0=No, 1=Yes, 9=DK]** _____

5A. Have you ever had any type of breast surgery? **[0=No (Skip to 6A), 1=Yes, 9=DK]** _____

5B. Can you tell me when you had your most recent breast surgery? _____ OR _____
Month/Year Age at Surgery

5C. At the time of your surgery, when you were not feeling well, say with a sore throat or other general illness, did you go to a primary care doctor at Henry Ford?

[0=No, 1=Yes, 9=DK] _____

6A. Have you ever had any other type of medical procedure where tissue was removed, like skin or a polyp?

[0=No (Skip to 7A), 1=Yes, 9=DK] _____

6B. Can you tell me what your most recent procedure was? _____

6C. And when did you have this procedure? _____ OR _____
Month/Year Age at Procedure

6D. Can you tell me the name and location of the medical facility or hospital where you had this procedure?

Name	City	State

7A. Have you ever been diagnosed with breast cancer?

[0=No (Skip to NO section below), 1=Yes, 9=DK] _____

7B. When were you diagnosed with breast cancer? _____ (Month/Year) OR _____ (Age at Diagnosis)

7C. Can you tell me the name and location of the medical facility or hospital where you were diagnosed?

Name	City	State

IF YES TO #7A:

"We are especially interested in learning more about breast cancer. We would like to contact you a year from now to ask you some additional questions about your health. For that reason, I would like to take a minute to confirm location information with you. "

IF NO TO #7A:

"We are very interested in the prevention of disease among women. We may be contacting you a year from now to ask you some additional questions about your health. For that reason, I would like to take a minute to confirm location information with you."

GO TO PRE-PRINTED DATA SHEET TO CONFIRM INFORMATION

8. If you have a vacation home or other residence, could you tell me the address, telephone number and time of year you at that residence?

[0=No Other Residence (Skip to 9), 1=Yes] _____

Street Address _____

City, State, Zip Code and Country _____

Phone (___ ___ ___) ___ ___ ___-___ ___ ___ ___

Time at Residence From (M/D): _____ / _____ To (M/D): _____ / _____

9. Can you tell me the names of two adults who live with you and what their relationship is to you?

[0=No/Lives Alone, 1=Yes, 2=Unwilling to State] _____

1. First and Last Name _____ Relationship_____

2. First and Last Name _____ Relationship_____

10. What is the name, address and telephone number of your current primary care physician or clinic?

[0=No Primary Care Physician, 1=Yes, 2=Unwilling to State] _____

Name of physician or clinic _____

Street Address _____

City, State, and Zip Code _____ _____

Phone (___ ___ ___) ___ ___ ___-___ ___ ___ ___

11. It would be great help to us if you could provide us with the names and addresses of two people who you do not live with that could give us your new address should you move. We would only contact these people if we were unable to reach you at your home address.

[0=No One Available, 1=Yes, 2=Unwilling to State] _____

1. Name of Contact _____

   Street Address _____

   City, State, and Zip Code _____ _____

   Phone (___ ___ ___) ___ ___ ___ -___ ___ ___ ___

2. Name of Contact _____

   Street Address _____

   City, State, and Zip Code _____ _____

   Phone (___ ___ ___) ___ ___ ___ -___ ___ ___ ___

CLOSING:
"That all the information that I need today. Thank you for taking the time to respond to these questions. Your cooperation in this women's health study is greatly appreciated. "

[Record the subjects willingness to be contacted in the future.] _____

1. Willing
2. Not willing
9. Don't Know

[Record who completed the Locator Form.] _____

1. Study Subject
2. Spouse
3. Offspring
4. Other (specify relationship) _____

BENIGN BREAST DISEASE SCREENING LOG - 19XX

15-Oct-97

N=XXX

Name	MRN	Pathology Report #	Pathology Report Date	# of Biopsies	Screen: 1=BBD 2=Cancer 3=N/A

BENIGN BREAST DISEASE COHORT LOG - 19XX

N=XXX

15-Oct-97

Name	MRN	Pathology Report #	Pathology Report Date	# of Biopsies	Slide Status: 1=Available 2=Pending 3=Not Available	# of Slides	Block Status: 1=Available 2=Pending 3=Not Available

BENIGN BREAST DISEASE PATHOLOGY REVIEW FORM

PLACE LABEL HERE:
MRN
Pathology # Specimen #
Date of Pathology Report

BIOPSY REVIEWER

☐0 No ☐1 Yes Usha Raju
☐0 No ☐1 Yes Richard Zarbo
☐0 No ☐1 Yes Sandra Wolman

FORM COMPLETION DATE

__/__/____

TYPE OF BIOPSY

☐1 Needle
☐2 Excision
☐3 Simple Mastectomy
☐4 Modified Radical Mastectomy
☐7 Other _____
☐9 Unknown

LOCALIZATION

☐0 No
☐1 Yes
☐9 Unknown

LOCATION OF BREAST BIOPSY

☐1 Left
☐2 Right
☐9 Unknown

BREAST QUADRANT

☐1 Upper Inner ☐4 Upper Outer
☐2 Lower Inner ☐5 Lower Outer
☐3 Central ☐9 Unknown

GROSS FINDINGS

☐1 No lesion
☐2 Cyst(s) ⇒ ☐1 Solitary ☐2 Multiple
☐3 Mass(es) ⇒ ☐1 Solitary ☐2 Multiple
Size of Largest Mass/Cyst ___ . ___ cm
☐7 Other _____
☐9 Unknown

MAMMARY EPITHELIAL TISSUE BIOPSY

☐0 No
☐1 Yes

MICROSCOPIC FINDINGS

SIMPLE APOCRINE METAPLASIA

PRESENT	FOCI	CALCIFICATIONS
☐0 No	☐1 1	☐0 No
☐1 Yes	☐2 2-5	☐1 Yes
	☐3 6+	

CYSTS

PRESENT	FOCI	CALCIFICATIONS
☐0 No	☐1 1	☐0 No
☐1 Micro Only	☐2 2-5	☐1 Yes
☐2 Macro	☐3 6+	

PERIDUCTAL MASTITIS/DUCT ECTASIA

PRESENT
- ☐ 0 No
- ☐ 1 Yes

CALCIFICATIONS
- ☐ 0 No
- ☐ 1 Yes

MASTITIS

PRESENT
- ☐ 0 No
- ☐ 1 Yes

FIBROSIS

PRESENT
- ☐ 0 No
- ☐ 1 Yes

CALCIFICATIONS
- ☐ 0 No
- ☐ 1 Yes

SQUAMOUS METAPLASIA

PRESENT
- ☐ 0 No
- ☐ 1 Yes

FOCI
- ☐ 1 1
- ☐ 2 2-5
- ☐ 3 6+

FIBROADENOMA

PRESENT
- ☐ 0 No
- ☐ 1 Yes

FOCI
- ☐ 1 1
- ☐ 2 2-5
- ☐ 3 6+

SIZE
___ . ___ cm

CALCIFICATIONS
- ☐ 0 No
- ☐ 1 Yes

BLOCK
_____

Associated Findings Within Lesion

HYPERPLASIA
- ☐ 0 No
- ☐ 1 Mild
- ☐ 2 Moderate/Florid

ADENOSIS
- ☐ 0 No
- ☐ 1 Yes

ADH
- ☐ 0 No
- ☐ 1 Yes

ALH
- ☐ 0 No
- ☐ 1 Yes

DCIS
- ☐ 0 No
- ☐ 1 Yes

LCIS
- ☐ 0 No
- ☐ 1 Yes

CYSTIC CHANGES
- ☐ 0 No
- ☐ 1 Yes

PLACE LABEL HERE

CELLULAR STROMA
- ☐ 0 No
- ☐ 1 Yes

SIMPLE ADENOSIS

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑$_0$ No	❑$_1$ 1	❑$_1$ ≤ 0.3 cm	❑$_0$ No	_____
❑$_1$ Mild	❑$_2$ 2-5	❑$_2$ 0.3 - 0.9 cm	❑$_1$ Yes	
❑$_2$ Moderate/Florid	❑$_3$ 6+	❑$_3$ 1.0 - 1.9 cm		
		❑$_4$ ≥ 2.0 cm		

Associated Findings Within Lesion

ADH	ALH	DCIS	LCIS
❑$_0$ No	❑$_0$ No	❑$_0$ No	❑$_0$ No
❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes

SCLEROSING ADENOSIS

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑$_0$ No	❑$_1$ 1	❑$_1$ ≤ 0.3 cm	❑$_0$ No	_____
❑$_1$ Mild	❑$_2$ 2-5	❑$_2$ 0.3 - 0.9 cm	❑$_1$ Yes	
❑$_2$ Moderate/Florid	❑$_3$ 6+	❑$_3$ 1.0 - 1.9 cm		
		❑$_4$ ≥ 2.0 cm		

Associated Findings Within Lesion

ADH	ALH	DCIS	LCIS
❑$_0$ No	❑$_0$ No	❑$_0$ No	❑$_0$ No
❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes

APOCRINE ADENOSIS

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑$_0$ No	❑$_1$ 1	❑$_1$ ≤ 0.3 cm	❑$_0$ No	_____
❑$_1$ Mild	❑$_2$ 2-5	❑$_2$ 0.3 - 0.9 cm	❑$_1$ Yes	
❑$_2$ Moderate/Florid	❑$_3$ 6+	❑$_3$ 1.0 - 1.9 cm		
		❑$_4$ ≥ 2.0 cm		

Associated Findings Within Lesion

ADH	ALH	DCIS	LCIS
❑$_0$ No	❑$_0$ No	❑$_0$ No	❑$_0$ No
❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes

PLACE LABEL HERE

HYPERPLASIA WITHOUT ATYPIA (USUAL TYPE)

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑0 No	❑1 1	❑1 ≤ 0.3 cm	❑0 No	_____
❑1 Mild	❑2 2-5	❑2 0.3 - 0.9 cm	❑1 Yes	
❑2 Moderate/Florid	❑3 6+	❑3 1.0 - 1.9 cm		
		❑4 ≥ 2.0 cm		

HYPERPLASIA WITHOUT ATYPIA (APOCRINE TYPE)

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑0 No	❑1 1	❑1 ≤ 0.3 cm	❑0 No	_____
❑1 Mild	❑2 2-5	❑2 0.3 - 0.9 cm	❑1 Yes	
❑2 Moderate/Florid	❑3 6+	❑3 1.0 - 1.9 cm		
		❑4 ≥ 2.0 cm		

ADH*

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑0 No	❑1 1	___ . ___ cm	❑0 No	_____
❑1 Yes	❑2 2-5		❑1 Yes	
	❑3 6+			

ALH*

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑0 No	❑1 1	___ . ___ cm	❑0 No	_____
❑1 Yes	❑2 2-5		❑1 Yes	
	❑3 6+			

PAPILLOMA

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑0 No	❑1 1	___ . ___ cm	❑0 No	_____
❑1 Yes	❑2 2-5		❑1 Yes	
	❑3 6+			

Associated Findings Within Lesion

HYPERPLASIA	ADENOSIS	ADH	ALH	DCIS	LCIS
❑0 No	❑0 No	❑0 No	❑0 No	❑0 No	❑0 No
❑1 Mild	❑1 Yes	❑1 Yes	❑1 Yes	❑1 Yes	❑1 Yes
❑2 Moderate/Florid					

PLACE LABEL HERE

RADIAL SCAR

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑$_0$ No	❑$_1$ 1	___ . ___ cm	❑$_0$ No	_____
❑$_1$ Yes	❑$_2$ 2-5		❑$_1$ Yes	
	❑$_3$ 6+			

Associated Findings Within Lesion

HYPERPLASIA	ADENOSIS	ADH	ALH	DCIS	LCIS
❑$_0$ No	❑$_0$ No	❑$_0$ No	❑$_0$ No	❑$_0$ No	❑$_0$ No
❑$_1$ Mild	❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes	❑$_1$ Yes
❑$_2$ Moderate/Florid					

LCIS*

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑$_0$ No	❑$_1$ 1	___ . ___ cm	❑$_0$ No	_____
❑$_1$ Yes	❑$_2$ 2-5		❑$_1$ Yes	
	❑$_3$ 6+			

DCIS*

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
❑$_0$ No	❑$_1$ 1	___ . ___ cm	❑$_0$ No	_____
❑$_1$ Yes	❑$_2$ 2-5		❑$_1$ Yes	
	❑$_3$ 6+			

INVASIVE CARCINOMA

PRESENT	FOCI	SIZE	BLOCK
❑$_0$ No	❑$_1$ 1	___ . ___ cm	_____
❑$_1$ Yes	❑$_2$ 2-5		
	❑$_3$ 6+		

PLACE LABEL HERE

LYMPHOCYTIC INFILTRATE

PRESENT	FOCI	CALCIFICATIONS	BLOCK
☐0 No	☐1 1	☐0 No	_____
☐1 Yes	☐2 2-5	☐1 Yes	
	☐3 6+		

Associated Findings With Lesion

NORMAL LOBULES	DUCT ECTASIA	DCIS	CYST(S)	OTHER _____
☐0 No	☐0 No	☐0 No	☐0 No	☐0 No
☐1 Yes	☐1 Yes	☐1 Yes	☐1 Yes	☐1 Yes

PHYLLODES TUMOR

PRESENT	CELLULAR STROMA	STROMAL OVERGROWTH	SIZE	MITOSIS
☐0 No	☐0 No	☐0 No	___.___ cm	_____ Count / 10 HPF
☐1 Yes	☐1 Yes	☐1 Yes		

HYPERPLASIA	MARGINS	TUMOR TYPE
☐0 No	☐0 Negative	☐1 Benign
☐1 Mild	☐1 Positive	☐2 Indeterminate
☐2 Moderate/Florid	Distance: ___.___ cm	☐3 Malignant

OTHER (please specify) _____

PRESENT	FOCI	SIZE	CALCIFICATIONS	BLOCK
☐0 No	☐1 1	___.___ cm	☐0 No	_____
☐1 Yes	☐2 2-5	☐9.9 N/A	☐1 Yes	
	☐3 6+			

Associated Findings Within Lesion

HYPERPLASIA	ADENOSIS	ADH	ALH	DCIS	LCIS
☐0 No	☐0 No	☐0 No	☐0 No	☐0 No	☐0 No
☐1 Mild	☐1 Yes	☐1 Yes	☐1 Yes	☐1 Yes	☐1 Yes
☐2 Moderate/Florid					

*ADH: Atypical Ductal Hyperplasia
ALH: Atypical Lobular Hyperplasia
LCIS: Lobular Carcinoma In Situ
DCIS: Ductal Carcinoma In Situ

PLACE LABEL HERE